Cell proliferation patterns during development of the equine placenta

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Placentation involves considerable growth and reorganization of both maternal and fetal tissues. In this investigation, immunohistochemical localization of the proliferation marker Ki-67 antigen was used to monitor cell division during placentation in mares. Endometrial biopsies were obtained from eight mares between day 14 and day 26 of pregnancy and from eight anoestrous mares that had been treated with various combinations of progesterone and oestrogen. Samples of endometrium and fetal membranes were obtained from 19 mares carrying normal horse conceptuses between day 30 and day 250 of gestation and from three failing extraspecific donkey-in-horse pregnancies. Proliferation in the superficial strata of the endometrium was increased by day 18 of gestation and this effect could be mimicked by supplementing with oestradiol benzoate during the last 6 days of a prolonged period (18–36 days) of progesterone administration. Fetal chorionic girdle cells were proliferating vigorously at days 30–32 of gestation, but stopped dividing after they invaded the endometrium, while the trophoblast cells of the allantochorion showed an increase in mitotic activity after day 38. The luminal epithelium of the endometrium started to proliferate only after the primary villi of the true epitheliochorial placenta had been formed, and during days 58–70 this effect was seen only in the pregnant horn in which placentation was further advanced. During the second half of gestation, most of the mitotic activity was confined to the periphery of the microcotyledons which were still growing. In the donkey-in-horse pregnancies, proliferation rates of the maternal and fetal epithelia at day 70 of gestation were markedly reduced in areas of heavy endometrial lymphocyte infiltration and poor placentation. These results provide a basis for further studies on factors that influence invasive and non-invasive placentation.

Introduction

Considerable growth and reorganization of both the endometrium and the conceptus membranes are required to form the complex structure of the placenta which is essential to support fetal growth throughout pregnancy. These processes are thought to be controlled by a combination of circulating steroid hormones and locally produced growth factors (Pollard, 1990; Weitlauf, 1994; Alsat *et al.*, 1995). Most investigations on placental growth patterns during pregnancy have been carried out in species with invasive placentae, especially rodents (Weitlauf, 1994) and humans (Kaufmann and Burton, 1994). However, there have been few studies on the actual proliferation patterns of the different types of cell involved and those that have been undertaken have sometimes produced contradictory results (Sands and

Dobbing, 1985; Simpson *et al.*, 1992). Nonetheless, it has been argued that even in humans in which the placenta is very complex, most placental pathology that causes fetal growth retardation is of a quantitative, rather than a qualitative, nature (Boyd, 1984). Quantitative growth abnormalities of the placenta are likely to be even more important as causes of fetal stunting or pregnancy failure in domestic animals that have a non-invasive epitheliochorial type of placenta, since in these species, it is the area of functional fetomaternal interface that becomes the most important factor in establishing adequate placental exchange (Amoroso, 1952).

Several unusual features of equine pregnancy make mares useful animals in which to study placental development. The young equine conceptus remains spherical and moves freely within the uterine lumen until day 16 after ovulation when it becomes fixed at the base of one of the uterine horns (van Niekerk, 1965; Ginther, 1979; Allen and Bracher, 1992). The yolk sac begins to regress from around day 22, while the allantois enlarges rapidly and fuses with the overlying

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Fig. 1. For legend see opposite.

chorion to form the allantochorion. In the region of abutment of the regressing yolk sac and growing allantois, the trophoblast cells start to differentiate to form the chorionic girdle (Ewart, 1897; van Niekerk, 1965; van Niekerk and Allen, 1975) and by day 35 the trophoblast has fully differentiated into its minor invasive (chorionic girdle) and major non-invasive (allantochorion) components. Between day 35 and day 38 the chorionic girdle cells become binucleate and invade the maternal endometrium to form endometrial cups (Allen *et al.*, 1973; Hamilton *et al.*, 1973) which secrete chorionic gonadotrophin (eCG) into the maternal circulation (Clegg *et al.*, 1954; Allen and Moor, 1972). The fetal cup cells elicit a maternal immune response and the resulting accumulation of leucocytes around each cup hastens their death and eventual sloughing from the endometrium between day 100 and day 120 (Allen, 1982). True epitheliochorial placentation does not commence until as late as day 40 of gestation when the non-invasive trophoblast of the allantochorion starts to interdigitate with the endometrium, thereby forming a stable villous contact. Shallow undulations are visible at day 45 and these develop into pronounced finger-like villi by day 60. The primary villi undergo secondary and tertiary branching during the next 60–80 days to give rise to the complex epitheliochorial microcotyledonary structure of the mature equine placenta (Samuel *et al.*, 1974; Steven, 1982).

Circumstantial evidence in horses highlights the importance of normal gross and microscopic development of the placenta for prenatal growth of the fetus and the successful outcome of pregnancy. Walton and Hammond

(1938) demonstrated profound fetal stunting when they inseminated Shetland pony mares with Shire horse semen, and a similar phenomenon occurs in twin pregnancies due to the limited placental surface area that is available to each of the two conceptuses (Jeffcott and Whitwell, 1973). Furthermore, it has been shown that abnormal growth at the microscopic level can lead to placental malnutrition. For example, Bracher *et al.* (1996) demonstrated that in aged subfertile mares suffering from chronic pathological changes of the endometrium, the chorionic villi and the microcotyledons are poorly developed, which results in fetal starvation. In addition, Allen *et al.* (1987) observed delayed and abnormal placental development as one of the causes of the high rate of abortion in mares carrying extraspecific donkey-in-horse pregnancies created by embryo transfer. Endometrial cups generally do not form in this type of equine pregnancy and >70% of them fail between day 80 and day 100 of gestation in conjunction with a pronounced leucocyte infiltration into the endometrial stroma of the surrogate mare and evidence of poor placentation (Allen *et al.*, 1987).

Characterization of normal placental growth in horses is a necessary prerequisite for further studies on placental insufficiency in this species. Gerstenberg *et al.* (1999) showed that immunohistochemical detection of the proliferation marker Ki-67 antigen is a valid and useful marker of cell proliferation patterns in the endometrium of the non-pregnant mare. The aim of the present study was to apply this technique to characterize growth patterns in the equine endometrium and placenta during normal gestation and in failing donkey-in-horse pregnancies.

Fig. 1. Immunohistochemical localization of the proliferation marker Ki-67 antigen during normal equine placentation. Positive nuclei are stained a dark brown colour. (a) On day 14 of gestation no significant mitotic activity can be detected in any types of cell of the endometrium. (b) By day 23 there is a marked increase in the proliferation rate of the epithelial cells in the gland necks and the fibroblasts of the stratum compactum. (c) Fetal membranes on day 32 showing the extremely high rate of mitotic activity in the specialized cells of the chorionic girdle (enlarged inset on the right) in marked contrast to the low proliferation rate in the non-invasive trophoblast cells of the allantochorion (enlarged inset on the left). Note that almost all the nucleated fetal red blood cells observed in the allantoic blood vessel (arrow) have strong positive staining, indicating active haemopoiesis or endogenous peroxidase activity. (d) Luminal surface of an endometrial cup with its overlying allantochorion on day 42 of gestation. The large fetal endometrial cup cells (arrows) do not show any positively stained nuclei. The few round nuclei with positive staining at the bottom of the photomicrograph are not surrounded by typical cup cell cytoplasm and probably represent proliferating lymphocytes. A number of mitotically active cells are evident in the intact layer of luminal epithelial cells that has already reformed above the endometrial cup. The proliferation rate in the non-invasive trophoblast cells of the allantochorion has also increased considerably (compare with part (c)). (e) Low power photomicrograph at the periphery of an endometrial cup on day 58 of gestation. The cup tissue is surrounded by a large number of lymphocytic round cells (arrows) that are clearly proliferating. Positively stained nuclei within the cup tissue belong either to lymphocytes or to proliferating epithelial cells of the enlarging endometrial glands trapped within the cup. Cross-sections of the enlarged glands situated in the area of inflammation surrounding the cup also show a high rate of mitotic activity. (f) Endometrium covered by allantochorion at day 42 of gestation. The proliferation rate in the non-invasive trophoblast cells has increased markedly at this stage, but very few positively stained nuclei are apparent in the luminal epithelium of the endometrium. The increased proliferation rate of stromal fibroblasts in the stratum compactum (bracket) persists but the rest of the endometrium is completely quiescent. Stromal macrophages, which are particularly numerous in this mare, show non-specific staining of the cytoplasm due to endogenous peroxidase activity (arrows). (g,h) Samples of placenta and endometrium from (g) the gravid and (h) the non-gravid horn recovered on day 70 of gestation. In the gravid horn, the primary villi have formed completely; there is evidence of early secondary branching and the mitotic rate of trophoblast cells in these areas has decreased considerably (compare with (f)). Simultaneously, the flattened luminal epithelial cells of the endometrium have started to proliferate. In the non-gravid horn, villi have not yet formed and the proliferation rates of the various types of cell are similar to those on day 42; namely, high in the non-invasive trophoblast cells of the allantochorion and low in the luminal epithelial cells of the endometrium. (i) Microcotyledons and underlying endometrium on day 200 of gestation. A basal rate of proliferation persists at the fetomaternal interface, both in the trophoblast cells and the luminal epithelium of the endometrium. Most of this mitotic activity is confined to the growing outer borders of the microcotyledons, while the central more established areas appear relatively quiescent. AC, allantochorion; CG, chorionic girdle; EC, endometrial cup; GN, gland necks; LE, luminal epithelial cells; MC, microcotyledons. Scale bars represent 100 μ m.

Fig. 2. Percentages of cells staining positively for Ki-67 antigen in the endometrial and placental tissues of normal intraspecies horse pregnancies grouped according to stage of gestation. Each vertical column of individual bars represents one biopsy specimen and the results for each type of cell are depicted as a separate bar showing the mean ($\pm$ SEM) percentage derived from counting the ratio of positively stained nuclei in five random areas on each section. At days 58 and 70 of gestation the extent of placental development and the cellular proliferation patterns were very different in the gravid horn (G) compared with the non-gravid horn (NG). The samples from the two horns were therefore evaluated separately and two columns represent the results from one animal. -, indicates that the sample was not available for analysis; a blank column represents a complete absence of positively stained nuclei in the five random areas of the biopsy.

Materials and Methods

Samples from pregnant animals

Samples of endometrium and fetal membranes were recovered from 25 mares carrying normal horse pregnancies

between day 14 and day 250 after ovulation (term 330–340 days). Up to day 32, unattached conceptuses and endometrial biopsies were recovered non-surgically from 11 mares by lavaging the uterus with 500–1500 ml PBS (pH 7.2) via a 14 mm endotracheal tube passed through the cervix with ($n = 3$) or without ($n = 8$) previous videoendoscopic

examination and rupture of the conceptus *in utero* as described by Bracher *et al.* (1992). Conceptuses that were ≤ 26 days were used for another experiment and were not available for examination. The collapsed membranes of conceptuses recovered between day 30 and day 32 were dissected under a stereomicroscope and areas of chorion and allantochorion on each side of a strip of chorionic girdle were selected for further processing. After the conceptus had been flushed from the uterus, rigid Yeoman-type biopsy forceps (Rocket Instruments, London) were passed through the cervix and a biopsy of endometrium was taken from the base of one of the uterine horns according to Ricketts (1975) and Bracher *et al.* (1992).

Surgical hysterotomy via ventral midline laparotomy was performed on ten mares between day 38 and day 70 of gestation. General anaesthesia was induced with 1.1 mg xylazine kg^{-1} (Rompun; Bayer plc, Bury St Edmunds) followed by 2.2 mg ketamine kg^{-1} (Ketaset; Ford Dodge Animal Health, Southampton) and was maintained with 2–4% halothane gas (Fluothane; Schering-Plough Animal Health, Welwyn Garden City). Six mares between day 100 and day 250 of pregnancy were killed with a free bullet and the uterus was removed and opened immediately. Samples of endometrium with the allantochorion attached were recovered from all these mares and biopsies of the endometrial cups were also taken on days 42, 58 and 70 of gestation.

Three extraspecific pregnancies were created by surgically transferring day 7 donkey embryos to three synchronized recipient mares that had previously aborted donkey fetuses (Allen *et al.*, 1987) to investigate abnormal placental growth in mares carrying donkey conceptuses. Samples of endometrium and allantochorion were collected surgically from each of the three mares at day 70 of gestation.

Hormone treatments

Eight seasonally anoestrous mares were given i.m. injections of progesterone in oil, with or without oestradiol benzoate, once a day to mimic the actions of the steroid hormones of pregnancy on the endometrium. The mares were diagnosed as being in anoestrus when serum progesterone concentrations had remained at basal values for 5 consecutive weeks and no follicles larger than 10 mm in diameter were detectable on their ovaries by ultrasound scanning. Four mares were treated for 15–30 days with 0.4 mg progesterone $\text{kg}^{-1} \text{ day}^{-1}$ alone to evaluate whether the duration of progesterone exposure affected the pattern of endometrial cell proliferation. The remaining four mares received the same dose of progesterone for 18–36 days and this was supplemented with i.m. injections of oestradiol benzoate at a dose rate of 0.02 mg $\text{kg}^{-1} \text{ day}^{-1}$ during the last 6 days of progesterone treatment to mimic the influence of oestrogens secreted by the conceptus. An endometrial biopsy was taken from each mare at the end of the treatment period using the method described earlier.

Sample processing

All the tissue samples were snap-frozen in isopentane over liquid nitrogen. They were stored at -70°C until

Fig. 3. Mean ($\pm$ SEM) percentages of trophoblast cells that stained positively for Ki-67 antigen in the three different areas of the placenta (chorion, chorionic girdle and allantochorion) of normal horse pregnancies. The results for each area are represented as individual bars for each of the three conceptuses recovered between days 30 and 32 of gestation.

cryosections were cut at a thickness of 6 μm and thaw-mounted onto slides coated with 3-aminopropyl-triethoxysilane (APTES; Sigma-Aldrich, Poole). The slides were air-dried briefly and then fixed for 5 min in a solution of 4% (w/v) paraformaldehyde in PBS at 4°C . After thorough rinsing in PBS and dehydration in an ethanol series, the slides were stored in 95% ethanol at 4°C until immunohistochemical staining.

Immunohistochemistry

Immunohistochemical detection of the proliferation marker Ki-67 antigen was used as described by Gerstenberg *et al.* (1999) to detect nuclei that were in the division stage of the cell cycle. The sections of endometrium and fetal membranes were rehydrated and immersed in Tris-buffered saline (TBS) for 15 min before blocking for 20 min with 3% (w/v) TBS-BSA. They were then incubated for 1 h at room temperature in a 1:200 dilution of the primary monoclonal antibody MIB 1 (Dianova, Hamburg) in 3% (w/v) TBS-BSA; negative control sections were incubated with 3% (w/v) TBS-BSA alone. The mouse IgG Vectastain ABC kit and a DAB peroxidase substrate kit (both from Vector Laboratories, Cambridge) were used to visualize Ki-67 immunoreactivity. The sections were lightly counterstained with Harris's haematoxylin for light microscopy.

Quantitative analysis

The proportion of cells showing Ki-67 immunoreactivity in any section was analysed as described by Gerstenberg

Fig. 4. Immunohistochemical staining for Ki-67 antigen in endometrial biopsies recovered from anoestrous mares treated with progesterone and oestrogen. (a) The endometrium showed no significant proliferative activity in any type of cell after a mare was injected i.m. with progesterone in oil ($0.4 \text{ mg kg}^{-1} \text{ day}^{-1}$) once a day for 30 days. (b) When a mare was given the same dose of progesterone in oil for 36 days but were also treated with i.m. injections of oestradiol benzoate ($0.02 \text{ mg kg}^{-1} \text{ day}^{-1}$) once a day for the last 6 days, the rate of cell division increased in the epithelium of the gland necks (GN) and the stromal cells of the stratum compactum (bracket). Only occasional positively stained nuclei are observed in the luminal epithelium (LE) and the deeper strata of the endometrium remain completely quiescent. Scale bars represent $100 \mu\text{m}$.

et al. (1999) by counting the number of positively stained nuclei for each type of cell in five random areas on each section at $\times 160$ – 400 magnification. In total, 30–40 luminal epithelial cells, 20–50 gland neck epithelial cells and 30–50 stromal cell nuclei were counted in each random field of endometrium. In the stratum spongiosum, 80–150 stromal cells and 130–250 gland epithelial cells were counted per field. In the placental membranes, the number of proliferating cells was determined in the trophoblast epithelium (a total of 25–45 cells counted in each random field) and in the stroma of the allantochorion (20–35 cells per field). From the individual percentages of proliferating cells in each of the five fields evaluated, the mean ($\pm \text{SEM}$) was calculated for each type of cell in each sample. The mean proliferating cell counts were grouped for the various stages of pregnancy or hormone treatments and differences were analysed by Student's *t* test when appropriate.

Results

Positive immunoreactivity for Ki-67 antigen was identifiable as dark rust-brown nuclear staining that was not detectable on the negative control sections. Hydrogen peroxide quenching was not included in the immunochemistry protocol to avoid tissue damage and this led occasionally to non-specific staining due to endogenous peroxidase activity in the stromal macrophages and siderocytes of some sections. However, this staining was cytoplasmic and lighter in colour and did not cause any problems in the histological analyses (see Fig. 1f). Representative examples showing the staining patterns for Ki-67 antigen in the different types of cell in the endometrium and placenta at the various stages of gestation are shown (Fig. 1a–i). The mean ($\pm \text{SEM}$)

percentages of proliferating cells counted for the different types of cell in each sample are shown (Fig. 2). Although the standard error bars demonstrate some variation in counts across the five fields of each biopsy section, this variation did not distract from the overall pattern observed. The number of positively stained cells in the deep stroma and epithelium of the fundic portions of the glands in the endometrium were consistently very small ($< 0.3\%$ and $< 3\%$, respectively) and did not vary throughout gestation; therefore, individual values are not presented for these types of cell.

At day 14 of gestation, the endometrium was completely quiescent and there was no significant mitotic activity in any of the types of cell. From day 18 onwards (Fig. 1a), an increase in mitotic activity occurred in the superficial stroma and the epithelium of the gland necks. This varied among individual mares, but was observed in all samples and became particularly pronounced by day 23 (Fig. 1b). Compared with day 14 of gestation, the cell proliferation rates on days 18–32 were significantly increased in the superficial stroma ($P = 0.01$) and in the epithelium of the gland necks ($P < 0.01$), but not in the luminal epithelium ($P = 0.08$). Cells in the stroma of the stratum compactum continued to proliferate at this increased rate until at least day 100 of gestation, but the mitotic rate in the gland neck epithelium decreased again (Fig. 2) and was significantly lower between day 32 and day 100 ($P < 0.01$).

Although considerable proliferative activity occurred in the stratum compactum of the endometrium between day 18 and day 32 of gestation, the trophoblast epithelium of the allantochorion remained quiescent, except in the area of the chorionic girdle (Fig. 1c). In the three conceptuses recovered on days 30–32, 70–90% of the chorionic girdle cells were proliferating, which was significantly higher ($P < 0.01$) than in the non-invasive trophoblast of the regressing chorion and

the expanding allantochorion in which <5% of the nuclei were mitotically active (Fig. 3). By day 42, the chorionic girdle cells had invaded the endometrium to form the endometrial cups and an intact layer of luminal epithelial cells, with some positively stained nuclei, had already reformed over the developing cups (Fig. 1d). The migrated girdle cells had already transformed into large endometrial cup cells and had ceased dividing (Fig. 1d,e). However, from this stage onwards an increasing number of lymphocytic round cells with high mitotic activity was observed around the periphery of each endometrial cup and some of these inflammatory cells had infiltrated into the cup tissue (Fig. 1e). The rate of proliferation was considerable in the epithelial cells of the dilated endometrial glands that were present inside the endometrial cups or had become involved in the surrounding inflammatory reaction (Fig. 1e). These were the only secretory gland cells observed to be multiplying to a significant extent throughout pregnancy.

Compared with days 30–32 of gestation, the proliferation rate of the non-invasive allantochorion increased markedly when true implantation commenced between day 38 and day 42. The percentage of proliferating cells in the non-invasive trophoblast of the allantochorion increased suddenly and highly significantly ($P < 0.01$) to around 40–50%, even before obvious interdigitation of the fetal membranes and endometrium could be observed (Figs 1f and 2). Mitotic activity in the opposing maternal epithelium remained negligible, but the relatively high rate of proliferation in the endometrial stroma beneath continued as before. By day 58, the pattern of proliferation had changed again. In those areas in which the primary villi had formed, and initial secondary and tertiary branching was already apparent, the percentage of positively stained nuclei in the trophoblast epithelium had decreased. However, coincidentally, the flattened luminal epithelial cells of the opposing endometrium started to proliferate. This effect of a significantly decreased rate of mitosis in the fetal trophoblast ($P < 0.01$) and a significantly increased rate of proliferation in the maternal epithelium ($P < 0.01$) was apparent only in the gravid uterine horn on days 58 and 70 of gestation. In the non-gravid horn, the primary villi had not formed completely and the proliferation pattern of the epithelial cells remained the same as that observed between day 38 and day 42 of gestation; namely, a high rate of proliferation in the fetal trophoblast and no significant mitotic activity in the maternal epithelium (Figs 1g,h and 2). By day 100 of gestation, the secondary and tertiary branches of the villi had given rise to the typical microcotyledonary architecture, and the rate of proliferation in both the fetal and maternal tissues had decreased to basal values (Fig. 2). Compared with the values for the gravid uterine horns on days 58 and 70, the cell proliferation rates were decreased significantly for both the trophoblast epithelium and the luminal epithelium ($P < 0.01$) on days 100 and 150. After day 100, most of the mitotic activity that continued in the interdigitating epithelial layers was confined to outer borders of the expanding microcotyledons that were still growing, while the inner established areas remained relatively quiescent (Fig. 1i).

The endometrial biopsies recovered from the four mares that had been treated with progesterone in oil alone for 15–30

Fig. 5. Percentages of nuclei that stained positively for Ki-67 antigen in the superficial strata of the endometrium in anoestrous mares treated with progesterone and oestrogen. Each vertical column of individual bars represents one animal and the results for each type of cell are depicted as an individual bar showing the mean ($\pm$ SEM) percentage derived from counting the ratio of positively stained nuclei in five random areas. Mares in group A were treated with i.m. injections of progesterone in oil ($0.4 \text{ mg kg}^{-1} \text{ day}^{-1}$) once a day for 15–30 days. Mares in group B received the same dose of progesterone in oil for 18–36 days, but the last 6 days of treatment were supplemented with i.m. injections of oestradiol benzoate ($0.02 \text{ mg kg}^{-1} \text{ day}^{-1}$) once a day. The mean results were grouped and highly significant differences ($P < 0.01$) were found for the gland neck epithelium and the superficial stroma, but not for the luminal epithelium, as denoted by the different letters (a,b).

days showed very few positively stained nuclei. The duration of progesterone exposure did not affect the results and all the biopsies were very similar in appearance to those recovered at day 14 of gestation (Fig. 4a). Conversely, the endometrial biopsies taken from the four mares in which the 18–36 days of progesterone treatment had been supplemented with oestradiol benzoate for the last 6 days, all showed a marked increase in proliferative activity in the epithelium of the gland necks and the superficial stroma, but there were only occasional positive cells in the luminal epithelium (Fig. 4b). The differences in proliferative activity between the two treatment groups were highly significant ($P < 0.01$) for the gland neck epithelium and superficial stroma (Fig. 5).

The extraspecific donkey-in-horse pregnancies that were removed on day 70 of gestation showed no evidence of endometrial cups. The allantochorionic villi were not well developed and they interdigitated poorly with the endometrium in some areas. The presence of both generalized diffuse and focal infiltrations of highly proliferative inflammatory round cells dominated the histological

Fig. 6. Section of an area of the interdigitating allantochorion and endometrium recovered from the gravid horn of a mare carrying a failing extraspecific donkey-in-horse pregnancy at day 70 of gestation. On the left, the placental villi are well developed and moderate proliferative activity is seen in both the fetal trophoblast cells and the flattened luminal epithelial cells of the endometrium (compare with Fig. 1g,h). On the right, the superficial stroma of the endometrium (bracket) shows heavy round cell infiltration and a large number of these lymphocytes are proliferating. In this area, the mitotic activities of the overlying fetal and maternal epithelia are significantly reduced and placental attachment is very poor. Whether the positively stained nuclei in the cross-sections of the dilated glands surrounded by the inflammatory reaction (arrows) indicate proliferation of the glandular epithelium or multiplying lymphocytes in the process of migrating into the gland lumen cannot be determined with certainty. Scale bar represents 20 μ m.

appearance of large areas of the endometrial stroma and made it impossible to identify and evaluate reliably the proliferation rate of endometrial fibroblasts. In areas in which placental villi had developed, the rates of proliferation in the trophoblast and endometrial epithelia were similar to those observed in conventional horse pregnancies. However, in regions of heavy superficial lymphocyte infiltration into the endometrial stroma, the proliferation rates of the luminal epithelium and the overlying trophoblast were significantly suppressed ($P < 0.01$) and placental attachment was particularly poor (Figs 6 and 7).

Discussion

Immunohistochemical localization of the nuclear proliferation marker Ki-67 antigen was found to be a useful technique for monitoring cellular proliferation patterns during placentation in mares. Very little proliferative activity was detectable in the endometrium in very early pregnancy and the biopsies from day 14 of gestation had the same appearance as those from late dioestrus (Gerstenberg *et al.*, 1999). Furthermore, prolonged treatment of anoestrous mares with exogenous progesterone alone failed to induce any detectable mitotic activity, similar to the results from mares in spontaneously prolonged dioestrus (Gerstenberg *et al.*, 1999). However, the percentage of proliferating cells in

Fig. 7. Percentages of nuclei that stained positively for Ki-67 antigen in the interdigitating fetal and maternal epithelia in the gravid uterine horns of three mares carrying failing extraspecific donkey-in-horse pregnancies (A, B, and C) on day 70 of gestation. In each animal, at least five areas in which the endometrium appeared normal (N), and another five areas in which the endometrium showed heavy lymphocytic round cell infiltration in the stratum compactum (L) were evaluated and the mean ($\pm$ SEM) results are represented as two separate bars for each animal and each type of cell. In all three mares, the percentage of proliferating cells in the areas showing leucocyte accumulation were significantly lower than in the areas of normal placental interdigitation ($P < 0.01$).

the stratum compactum had increased significantly by day 18 of gestation and remained high thereafter. The equine conceptus produces significant quantities of oestrogens from as early as day 12 of gestation (Zavy *et al.*, 1984). The same pattern of an increased proliferation rate in the superficial strata of the endometrium was observed in the present study when oestradiol benzoate was administered to anoestrous mares that had been treated with exogenous progestagens. These results indicate that conceptus-derived oestrogens are likely to be the cause of the increased proliferative activity that occurs in the gland neck epithelium and superficial stroma of the endometrium during early pregnancy. The stromal proliferation in mares is reminiscent of the stromal mitosis induced in rodents and women by luteal oestrogens in preparation for implantation and decidualization (Psychoyos, 1986).

Although most of the trophoblast epithelium proliferated at a low basal rate of $< 5\%$ positive nuclei before implantation, the invasive chorionic girdle showed a markedly increased growth rate (70–90% of nuclei staining positively for Ki-67 antigen) between day 30 and day 32 of gestation. Ewart (1897) first described the histological appearance of the equine chorionic girdle and van Niekerk (1965), Allen *et al.* (1973) and Enders and Liu (1991a) all reported on the many mitotic figures visible in the finger-like folds or villi of modified trophoblast cells that comprise the girdle. Stewart *et al.* (1995) proposed that hepatocyte growth

factor-scatter factor (HGF-SF), secreted by the allantoic mesoderm, may act as the local mitogenic stimulus for the formation of the chorionic girdle. The findings of the present study support the observation (Allen *et al.*, 1973; Hamilton *et al.*, 1973; Enders and Liu, 1991a,b) that the girdle cells have stopped dividing by the time they have migrated into the endometrium. This cessation of proliferative activity upon invasion of the maternal tissues is similar to the situation in the human placenta in which fetal cells continuously leave the proliferating cytotrophoblastic columns and shell during the first trimester of pregnancy (Genbacev *et al.*, 1992; Kaufmann and Burton, 1994), but then cease to show mitotic activity as they invade the maternal decidua (Bulmer *et al.*, 1988; Loke, 1990).

The girdle cells pass through and in between the luminal epithelial cells of the endometrium to reach the basal lamina, and these become displaced and destroyed in the process (Allen *et al.*, 1973; Enders and Liu, 1991b). The localized mitotic activity observed in the maternal epithelium above the newly formed endometrial cups at day 42 of gestation probably represents the repair process of the surface epithelium as described by Allen (1982). Some girdle cells pass down the endometrial glands by migrating between the glandular epithelial cells and their basal lamina before entering the endometrial stroma (Allen *et al.*, 1973; Allen, 1979). According to Enders and Liu (1991b) the girdle cells do not displace the gland cells and the continuity of the glandular epithelium is never disrupted. Therefore, in the present study, the observation of a high degree of proliferation of the epithelial cells of the dilated endometrial glands persisting within the endometrial cup tissue and caught up in the surrounding inflammatory reaction was unexpected. Indeed, there was no significant proliferative activity in the glandular epithelium throughout the remainder of the endometrium at any stage of gestation, which is similar to the situation described in pregnant women (Bulmer *et al.*, 1988). The accumulation of leucocytes around the endometrial cups has been described in detail (Allen, 1982; Kydd, 1989; Grünig *et al.*, 1995) and probably involves cell trafficking in response to chemotactic stimuli (Grünig *et al.*, 1995). Nevertheless, the results of this study demonstrated that multiplication of the local resident lymphocyte population is also involved in the process.

Between day 38 and day 42 of gestation, when the allantochorion started to interdigitate with the endometrium, the trophoblast showed a sudden increase in proliferative activity, which was maintained until the primary villi became established, but then decreased during the period of secondary and tertiary branching of the villi. The cells of the luminal epithelium of the maternal endometrium did not proliferate significantly during indentation by the primary villi so that they became stretched and flattened. Thereafter, they showed a brief period of mitotic activity until the complex microcotyledonary structure was established around day 100 of gestation. The equine conceptus implants initially at the base of one of the uterine horns and the growing placenta gradually extends into the uterine body and then the contralateral horn from day 50 onwards (Douglas and Ginther, 1975; Marrable and Flood, 1975). Thus, the primary villi are fully developed in the gravid horn

by day 60–70, but they lag behind considerably in the non-gravid side, so that the cellular proliferation patterns of the maternal and fetal epithelia differ considerably between the two horns, and are determined by the stage of placental development rather than the stage of gestation.

Studies on obligate and facultative delayed implantation (reviewed by Aitken, 1979), and especially the crossed transfer experiments of 'dormant' and 'active' rat blastocysts and uteri undertaken by Dickmann and De Feo (1967) have demonstrated that it is the uterine environment that largely determines the onset of implantation. Thus, in mares, a uterine factor, possibly epidermal growth factor (EGF), may provide the stimulus for the sudden increase in mitotic activity observed in the allantochorion as it starts to interdigitate with the endometrium. EGF is a potent mitogen (Fisher and Lakshmanan, 1990) that has been implicated in placentalation in many species (Lacroix and Kann, 1993). In mares, the concentration of EGF mRNA increases markedly in the epithelium of the endometrial glands after day 38 of gestation (Stewart *et al.*, 1994; Lennard *et al.*, 1995). Furthermore, since, in mares, EGF continues to be expressed at high concentrations in the endometrium until at least day 250 of gestation (Lennard *et al.*, 1998), it may continue to provide a stimulus to term.

The abnormalities of placentalation in the extraspecific donkey-in-horse model of pregnancy failure have been described and the maternal lymphocytic immune response has been characterized in detail (Allen, 1982; Allen *et al.*, 1987; Kydd, 1989). In the failing donkey-in-horse pregnancies examined in the present study, the proliferation rates of the endometrial epithelium and the overlying trophoblast were both reduced markedly in regions in which the superficial endometrial stroma was heavily infiltrated with lymphocytes, and the placental villi were particularly poorly developed in these areas. Although the underlying mechanisms for this detrimental effect remain speculative, EGF gene expression in the secretory epithelium of the endometrial glands is depressed in those glands that are surrounded by a heavy infiltration of lymphocytes (Lennard *et al.*, 1998). This deficiency may contribute to the decreased cellular proliferation observed in the overlying maternal and fetal epithelia. However, an alternative explanation could be that the accumulated lymphocytes produce inflammatory products or cytokines that either damage the overlying epithelial cells so that they become unresponsive to a mitogenic factor or that have a direct antiproliferative action.

This study constitutes the first comprehensive description of cell proliferation patterns during formation of the placenta in a domestic animal species and includes preliminary evidence on the roles of steroid hormones and growth factors at different stages of placentalation. It also provides a basis for further studies on the factors controlling invasive and non-invasive placentalation in equids and other species.

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